Phylogenetic relationships of the genus *Kluyvera*: transfer of *Enterobacter intermedius* Izard *et al.* 1980 to the genus *Kluyvera* as *Kluyvera intermedia* comb. nov. and reclassification of *Kluyvera cochleae* as a later synonym of *K. intermedia*

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In order to assess the relationship between the genus *Kluyvera* and other members of the family *Enterobacteriaceae*, the 16S rRNA genes of type strains of the recognized *Kluyvera* species, *Kluyvera georgiana*, *Kluyvera cochleae*, *Kluyvera ascorbata* and *Kluyvera cryocrescens*, were sequenced. A comparative phylogenetic analysis based on these 16S rRNA gene sequences and those available for strains belonging to several genera of the family *Enterobacteriaceae* showed that members of the genus *Kluyvera* form a cluster that contains all the known *Kluyvera* species. However, the type strain of *Enterobacter intermedius* (ATCC 33110^T) was included within this cluster in a very close relationship with the type strain of *K. cochleae* (ATCC 51609^T). In addition to the phylogenetic evidence, biochemical and DNA–DNA hybridization analyses of species within this cluster indicated that the type strain of *E. intermedius* is in fact a member of the genus *Kluyvera* and, within it, of the species *Kluyvera cochleae*. Therefore, following the current rules for bacterial nomenclature and classification, the transfer of *E. intermedius* to the genus *Kluyvera as Kluyvera intermedia* comb. nov. is proposed (type strain, ATCC 33110^T = CIP 79.27^T = LMG 2785^T = CCUG 14183^T). Biochemical analysis of four *E. intermedius* strains and one *K. cochleae* strain independent of the respective type strains further indicated that *E. intermedius* and *K. cochleae* represent the same species and are therefore heterotypic synonyms. Nomenclatural priority goes to the oldest legitimate epithet. Consequently, *Kluyvera cochleae* Müller *et al.* 1996 is a later synonym of *Kluyvera intermedia* (Izard *et al.* 1980) Pavan *et al.* 2005.

INTRODUCTION

Despite their great medical and economical importance, the bacteria included in the family *Enterobacteriaceae* are still poorly understood phylogenetically. Furthermore, there is evidence suggesting the need for extensive revision of the taxonomic relationships among genera and species within this family. For example, based on 16S rRNA trees (Drancourt *et al.*, 2001), it has been reported that the genus *Klebsiella* is heterogeneous and composed of species which form three clusters that include members of other genera.

Kluyvera is a genus of small rod-shaped bacteria, thus conforming to the general definition of the family *Enterobacteriaceae* (Holt *et al.*, 1994). Bacteria of this genus are mainly grouped in four known species, *Kluyvera ascorbata*,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *K. cochleae* ATCC 51609^T, *K. georgiana* ATCC 51603^T, *K. cryocrescens* ATCC 33435^T, *K. ascorbata* ATCC 33433^T and *Enterobacter intermedius* ATCC 33110^T are AF047187, AF047186, AF310218, AF310219 and AF310217, respectively.

A phylogenetic tree showing that members of the genus *Enterobacter* are intertwined with members of other genera is available as supplementary material in IJSEM Online.

Kluyvera cryocrescens, *Kluyvera cochleae* and *Kluyvera georgiana* (Farmer *et al.*, 1981; Müller *et al.*, 1996).

The present study was undertaken to gain insight into the phylogenetic relationships among species within the genus *Kluyvera* and between the genus *Kluyvera* and related genera within the family *Enterobacteriaceae*, using 16S rRNA gene-based trees, DNA–DNA hybridization analysis and phenotypic characterization.

METHODS

Bacterial strains. Culture collection strains used in this study were *K. cochleae* ATCC 51609^T (=CDC 9514-94^T=DSM 9406^T) and ATCC 51717 (CDC 9532-94=DSM 9407), *K. georgiana* ATCC 51603^T (=CDC 2891A-76^T=CDC 2891-76^T=DSM 9409^T), *K. ascorbata* ATCC 33433^T (=CDC 0648-74^T), *K. cryocrescens* ATCC 33435^T (=CDC 2065-78^T) and *Enterobacter intermedius* ATCC 33110^T (=CUETM 77-130^T=CIP 79.27^T=Gavini E 86^T). Strains 77/123, 77/136 and 77/139 were received from D. Old (Ninewells Hospital and Medical School, Dundee, UK) and strain CDC 9011-82 was received from the Centers for Disease Control and Prevention (Atlanta, GA, USA) as *Enterobacter intermedius*. Culture was performed on Luria–Bertani medium at 35 °C under aerobic conditions.

Biochemical studies. *Enterobacter intermedius* and *Kluyvera* strains were characterized phenotypically using a battery of 43 biochemical tests in conventional media. These tests included: triple-sugar iron agar reactions; pigmentation (25 °C); motility; production of cytochrome oxidase, nitrate reductase and indole; growth in KCN broth; urea hydrolysis (Christensen's); utilization of malonate, citrate (Simmon's), acetate and mucate; production of β-galactosidase (ONPG) and phenylpyruvic acid (phenylalanine deaminase); lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase (Møeller's) activities; elaboration of acetylmethylcarbinol (Voges–Proskauer); degradation of gelatin, corn oil (lipase), DNA and polypectate (25 °C); and aesculin hydrolysis (broth). Carbohydrate fermentation reactions were performed in extract broth against 1 % solutions of the following carbohydrate or carbohydrate-like compounds: adonitol, amygdalin, L-arabinose, D-arabitol, cellobiose, dulcitol, methyl α-D-glucopyranoside, D-glucose, glycerol, *myo*-inositol, lactose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, D-sorbitol, salicin, sucrose, trehalose and D-xylose. All of these tests have been described previously (Abbott *et al.*, 1992, 2003; Janda *et al.*, 1996). Unless otherwise specified, tests were incubated at 35 °C for 2–4 days (7 days for carbohydrate fermentation and extracellular enzymes) and final results were recorded. Biochemical reactions presented in Tables 1 and 2 are at 48 h incubation.

Bacterial DNA extraction and analysis. Bacterial DNA was extracted by standard procedures and analysed using electrophoresis in agarose gels of serial dilutions of *K. cochleae* ATCC 51609^T and *Enterobacter intermedius* ATCC 33110^T DNA of equal concentration and Southern blot capillary transfer to nylon membranes under alkaline conditions (Sambrook *et al.*, 1989).

DNA–DNA hybridization. The genetic relatedness among members of the genus *Kluyvera* was determined by DNA–DNA hybridization on nylon membranes (Johnson, 1991). Serial dilutions of cell suspensions of equal OD_{570} values in denaturation solution (0·5 M NaOH/1·0 M NaCl) were applied to nylon membranes (Pall Biodyne). The membranes were then neutralized with 1·5 M NaCl/0·5 M Tris/HCl (pH 8) and fixed by UV radiation for 15 min. Pre-hybridization (2 h) was performed at 65 °C in a buffer containing 0·15 M NaCl, 1 % SDS and 0·3 % non-fat dried milk, and hybridization (18 h) was performed at 65 °C in a buffer containing 0·03 M NaCl, 1 % SDS and 0·3 % non-fat dried milk. The hybridization probe was chromosomal DNA from the indicated strain digested with *Alu*I endonuclease and ^{32}P-labelled with the Random Primer DNA Labelling System (Gibco, Life Technologies). The results were scored first by autoradiography and then by cutting the spots and measuring the radiation in a scintillation counter (Beckman). Hybridization levels were calculated as described by Johnson (1991).

Table 1. Key reactions for *Enterobacter intermedius*, *K. cochleae*, *K. georgiana*, *K. ascorbata* and *K. cryocrescens*

1, *E. intermedius* ATCC 3310^T; 2, *K. cochleae* ATCC 51609^T; 3, *K. georgiana* ATCC 51603^T; 4, *K. ascorbata* ATCC 33433^T; 5, *K. cryocrescens* ATCC 33435^T. Only variable results are shown in the table. The following tests gave identical results for the five type strains: triple-sugar iron (24 h) (acid/acid+gas); motility (+); urea hydrolysis (−); ONPG (24 h) (+); utilization of citrate (+) and acetate (+); degradation of mucate (+), DNA (−), corn oil (−) and gelatin (−); polypectate (+); Møeller's reaction, arginine (−) and ornithine (+); phenylpyruvic acid test (−); fermentation of L-arabinose (+), D-glucose (+), L-rhamnose (+), D-xylose (+), cellobiose (+), lactose (+), maltose (+), sucrose (+), trehalose (+), raffinose (+), adonitol (−), *myo*-inositol (−), D-mannitol (+), salicin (+), melibiose (+), amygdalin (+) and D-arabitol (−); aesculin hydrolysis (+); pigmentation (−); growth in KCN broth (+). Reactions are reported at 48 h.

Test	1	2	3	4	5
Formation of indole	−	−	+	+	+
Utilization of malonate	+	+	−	+	−
Voges–Proskauer	+	+	−	−	−
Lysine (Møeller's reaction)	−	−	+	+	−
Fermentation of:					
Dulcitol	+	+	+	−	−
Erythritol	−	−	−	−	−
Glycerol*	+G	+G	+^{w}	+^{w}	+G
D-Sorbitol	+	+	−	−	−
Ascorbate	+	+	+	+	−
Growth on CIN†	+	+	+	+	−

*G, Gas production; ^{w}, weakly acidic.
†CIN, Cefsulodin/irgasan/novobiocin.

Sequencing of the 16S rRNA gene and phylogenetic analysis. DNA from bacterial strains was purified (Sambrook *et al.*, 1989), and the 16S rRNA genes were amplified by PCR using the bacterial primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′), corresponding to positions 8–27 of forward *Escherichia coli* numbering, and 1492r (5′-GGTTACCTTGTTACGACTT-3′), corresponding to positions 1510–1492 of reverse *Escherichia coli* numbering. The following temperature programme was used: 94 °C for 5 min, 30 cycles of 94 °C for 60 s, 60 °C for 60s and 72 °C for 60 s, followed by a final 7 min incubation at 72 °C. The PCR product was purified using GFX-PCR DNA and a gel band purification kit (Amersham Biosciences) and sequenced completely by using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) and an ABI Prism 377 DNA Sequencer (Perkin-Elmer). The

Table 2. Phenotypic comparison between *K. cochleae* and *Enterobacter intermedius* strains

1, *K. cochleae* ATCC 51716; 2, *K. cochleae* ATCC 51717; 3, *Enterobacter intermedius* ATCC 33110^T; 4, *E. intermedius* CDC 9011-82; 5, *E. intermedius* 77-123; 6, *E. intermedius* 77-136; 7, *E. intermedius* 77-139; 8, *E. intermedius* 77-140. Only variable results are shown in the table. The following tests gave identical results for all the strains: triple-sugar iron (acid/acid + gas); motility (+); pigmentation (−); hydrolysis of urea (−) and aesculin (+); utilization of citrate (+) and malonate (+); growth in KCN broth (+); formation of indole (−), acetylmethylcarbinol (+) and phenylpyruvic acid (−); Møeller's reactions, lysine (−), arginine (−) and ornithine (+); degradation of mucate (+), DNA (−), corn oil (−) and gelatin (−); nitrate reductase (+); fermentation of L-arabinose (+), D-glucose* (+), L-rhamnose (+), D-xylose (+), cellobiose* (+), lactose* (+), maltose (+), trehalose (+), raffinose (+), adonitol (−), glycerol* (+), *myo*-inositol (−), D-mannitol (+), D-sorbitol (+), salicin (+), melibiose (+), D-arabitol (−) and methyl α-D-glucopyranoside (+). *, Gas determined. Biochemical reactions are reported at 48 h; (+), delayed positive reactions (>48 h).

Test	1	2	3	4	5	6	7	8
ONPG	+	−	+	+	+	+	+	+
Utilization of:								
Acetate	+	+	+	+	(+)	+	+	+
Degradation of:								
Polypectate	+	+	+	+	(+)	+	(+)	(+)
Fermentation of:								
Sucrose	+	+	+	+	(+)	(+)	(+)	+
Dulcitol	+	+	(+)	(+)	+	+	+	+
Amygdalin	(+)	+	(+)	(+)	(+)	(+)	(+)	(+)

obtained 16S rRNA gene sequences were aligned with those of type strains of bacterial genera related to the genus *Kluyvera* available in the EMBL and Ribosomal Database Project libraries (Maidak *et al.*, 1997) by using the CLUSTAL W program (Thompson *et al.*, 1994) with default parameters and optimized using a multiple sequence alignment editor (Galtier *et al.*, 1996). Phylogenetic trees were constructed by both the neighbour-joining distance method (Kimura two-parameter model and jumble option) and the parsimony character method, using programs contained in the PHYLIP package (Felsenstein, 1989). The stability of the relationships was assessed by bootstrapping (1000 replicates), with programs included in the same package. The sequence of *Aeromonas hydrophila* ATCC 7966^T (GenBank/EMBL/DDBJ accession no. X74677) was used as an outgroup to establish the root of the tree.

RESULTS AND DISCUSSION

In order to understand the phylogenetic relationships of members of the genus *Kluyvera* with other members of the family *Enterobacteriaceae*, the 16S rRNA genes of the type strains of *K. cochleae* (ATCC 51609^T), *K. georgiana* (ATCC 51603^T, *K. cryocrescens* (ATCC 33435 ^{T}) and *K. ascorbata* (ATCC 33433 ^{T}) were sequenced. Also, for reasons to be discussed below, the 16S rRNA gene of the type strain of *Enterobacter intermedius* (ATCC 33110^T) was sequenced. A phylogenetic tree, constructed using the neighbour-joining distance method, of members of the genus *Kluyvera* and related genera is shown in Fig. 1.

As can be observed, the four members of the genus *Kluyvera* clustered together in the tree. However, the type strain of *Enterobacter intermedius* was included in the *Kluyvera* cluster very close to the type strain of *K. cochleae* (bootstrap value 100 %). A tree constructed using the parsimony character method was in complete agreement with this (not shown). It should be also pointed out that, according to the base composition of its 16S rRNA gene, *Enterobacter intermedius* is distantly related to *Enterobacter cloacae* (similarity value 97·4 %), the type species of the genus *Enterobacter*, in comparison with its relationship to *K. cochleae* (similarity value 99·9 %). This strongly suggested that, if there was a misclassification of the *Enterobacter intermedius* type strain, many (if not all) strains of the same species may also be misclassified. A phenotypic analysis (Table 1) showed that *Enterobacter intermedius* ATCC 33110^T is remarkably similar to the type strain of *K. cochleae* and that both strains are Voges–Proskauer-positive, differing from the type strains of the other *Kluyvera* species. Furthermore, Table 2 shows that this remarkable similarity is not exclusive to the type strains but can be extended to other available independently isolated strains of these two species. The DNA relatedness among the type strains of species in the genus *Kluyvera* and the type strain of *Enterobacter intermedius* was also studied (Table 3). As can be observed, this DNA relatedness was higher than the current minimal standard (70 % relatedness) accepted for the phylogenetic definition of a species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994) when labelled DNA from *Enterobacter intermedius* was used as a probe. However, the DNA relatedness was about 59 % when labelled DNA from *K. cochleae* was used as a probe. This difference may be due to the presence of extrachromosomal DNA in one or both of the reference strains. In agreement with this, Fig. 2 shows that cells of the type strain of *Enterobacter intermedius* have several high-copy-number plasmids, while the cells of the type strain of *K. cochleae* apparently do not have extrachromosomal elements. Using chromosomal DNA of the type strain of *Enterobacter intermedius* extracted from gels, the DNA relatedness with the DNA of the type strain of *K. cochleae* increased to more than 70 % (not shown). Thus, DNA–DNA hybridization assays indicate that the type strains of these bacterial species are included within the same species.

Taken together, these results indicate that *Enterobacter intermedius* phenotypically and genotypically is a member of the genus *Kluyvera*. According to Rules 27(2) and 27(3) of the Bacteriological Code, we formally propose to transfer *Enterobacter intermedius* to the genus *Kluyvera* as *Kluyvera intermedia* comb. nov. The species name *intermedia* is proposed because Latin feminine adjectives must agree in gender with the feminine generic name *Kluyvera* (Farmer *et al.*, 1981). DNA–DNA hybridization assays indicate that

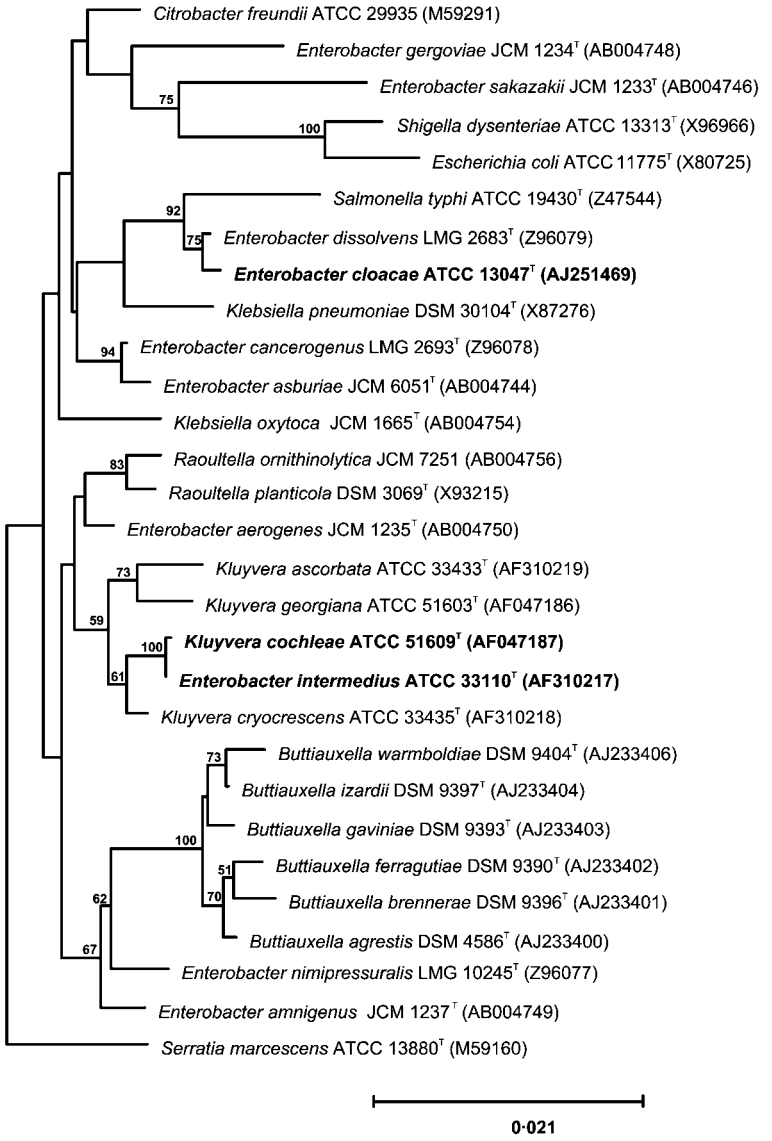

Fig. 1. Phylogenetic tree derived from the analysis of the 16S rRNA gene sequences of members of the family *Enterobacteriaceae* according to the neighbour-joining distance method. The Kimura two-parameter model was used to correct the distances for multiple substitutions at a site. Bootstrap values are from 1000 replications and only those greater than 50 % are shown. Bar, substitutions per nucleotide position.

Table 3. DNA relatedness (%) among type strains of species of the genus *Kluyvera* and the type strain of *Enterobacter intermedius*

1, *K. ascorbata* ATCC 33433^T; 2, *K. cochleae* ATCC 51609^T; 3, *K. cryocrescens* ATCC 33435^T; 4, *K. georgiana* ATCC 51603^T; 5, *Enterobacter intermedius* ATCC 33110^T; 6, *Escherichia coli* ATCC 11775^T (unrelated control). Results are expressed as the mean of three experiments; standard error <3 %.

Labelled DNA probe	1	2	3	4	5	6
K. ascorbata	100	16	15	17	19	11
K. cochleae	20	100	26	16	**59**	12
K. cryocrescens	19	23	100	22	29	15
K. georgiana	17	18	25	100	14	13
Enterobacter intermedius	21	**89**	30	13	100	16
Escherichia coli	13	13	14	12	14	100

K. cochleae and *K. intermedia* are heterotypic synonyms. Rule 42 of the Bacteriological Code requires the oldest legitimate epithet be retained. Thus, the oldest legitimate epithet for these taxa is that of Izard *et al.* (1980). Therefore, *K. cochleae* Müller *et al.* 1996 is a later synonym of *K. intermedia* (Izard *et al.* 1980) Pavan *et al.* 2005.

It is also worth noting that the 16S rRNA gene-based tree presented here strongly suggests that members of the genera *Kluyvera* and *Buttiauxella*, as defined by phenotypic assays, are monophyletic, in agreement with the conclusion of a previous report (Spröer *et al.*, 1999). In contrast, members of the genus *Enterobacter* are intertwined with members of other genera (see Fig. A, available as supplementary material in IJSEM Online), a fact also suggested by trees constructed using *groE* genes (Harada & Ishikawa, 1997). This indicates the need for an extensive revision of the phenotypic criteria to classify bacteria within the genus *Enterobacter*.

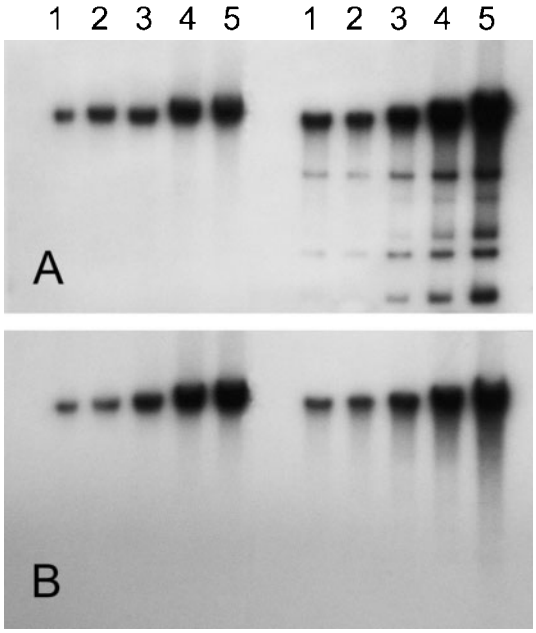

Fig. 2. Southern blot analysis of total DNA extracted from cells of *K. cochleae* ATCC 51609^T and *Enterobacter intermedius* ATCC 33110^T. (A) Results of the Southern blot analysis when the probe was labelled DNA from *Enterobacter intermedius*. (B) Results of the Southern blot analysis when the probe was labelled DNA from *K. cochleae*. Lanes 1, 2, 3, 4 and 5 correspond to 2·5, 1·25, 0·62, 0·31 and 0·15 μg of total DNA, respectively.

There is a good correlation between the results of DNA–DNA hybridization studies presented previously (Farmer *et al.*, 1981) and the results of the phylogenetic analysis presented herein. In both cases, *Klebsiella*, *Enterobacter* and *Citrobacter* species are close relatives of *Kluyvera* species. *Salmonella*, *Escherichia*, *Shigella* and *Erwinia* species are intermediate and *Proteus* and *Yersinia* species are the most distant relatives among the species analysed in both studies. In the Farmer *et al.* (1981) analysis, the large heterogeneity of the *Enterobacter* species observed in our study was also evident.

In conclusion, the results presented herein indicate that there is good agreement between the grouping of species of the genus *Kluyvera* by 16S rRNA gene-based phylogenetic analysis and phenotypic clustering, and that strains of *Enterobacter intermedius* should be reclassified as proposed since they are by phylogenetic, phenotypic and DNA–DNA relatedness criteria members of the genus *Kluyvera*. On the other hand, it is clear from this study that extensive studies are necessary to bring about coherence within the genus *Enterobacter*.

Description of *Kluyvera intermedia* comb. nov.

Kluyvera intermedia (in.ter.me′di.a. L. adj. *intermedia* intermediate).

Basonym: *Enterobacter intermedius* Izard *et al.* 1980.

The description is that given by Izard *et al.* (1980). Some characteristics are as follows. Cells are straight rods, 0·5–0·7 × 2–3 μm, Gram-negative, motile by scant peritrichous flagella. Facultatively anaerobic and chemo-organotrophic, having both a respiratory and a fermentative type of metabolism. Colonies are circular, convex, greyish and smooth on nutrient agar, with growth at 30 and 37 °C. Catalase-positive. Oxidase-negative. Nitrate reductase-positive. Indole-negative. Voges–Proskauer-positive. Acid produced from amygdalin, L-arabinose, cellobiose, dulcitol, D-glucose, glycerol, lactose, maltose, D-mannitol, melibiose, raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, trehalose and D-xylose. Negative for urea hydrolysis. ONPG-positive. Grows in KCN broth. Utilizes citrate and acetate. Does not utilize adonitol, arabitol, erythritol or *myo*-inositol. Does not degrade gelatin, DNA (DNase-negative) or corn oil (lipase-negative), but degrades mucate. Arginine dihydrolase-negative. Does not form phenylpyruvic acid. Does not produce pigment at 25 °C. Polypeptate-positive. Isolated from molluscs, surface water, soil and a variety of human samples including stool, blood, wounds, bile and a gall bladder.

The type strain is ATCC 33110^T (=CIP 79.27^T=LMG 2785^T=CCUG 14183^T).

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